Dionex IonPac AmG-3µm C18 Columns

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For Research Use Only. Not for use in diagnostic procedures.

Product Manual

for

Dionex IonPac AmG-3µm C18 Analytical Column

4 × 150 mm (Item # 302693)

Dionex IonPac AmG-3µm C18 Guard Column

4 × 30 mm (Item # 302694)

For Research Use Only. Not for Use in Diagnostic Procedures.

Revision History:

Revision 01, May, 2017, Original Publication.

Safety and Special Notices

Make sure you follow the precautionary statements presented in this guide. The safety and other special notices appear in boxes.

Safety and special notices include the following:

 **SAFETY**	***Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.***
 **WARNING**	***Indicates a potentially hazardous situation which, if not avoided, could result in damage to equipment.***
 **CAUTION**	***Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. Also used to identify a situation or practice that may seriously damage the instrument, but will not cause injury.***
 **NOTE**	***Indicates information of general interest.***
IMPORTANT	***Highlights information necessary to prevent damage to software, loss of data, or invalid test results; or might contain information that is critical for optimal performance of the system.***
Tip	***Highlights helpful information that can make a task easier.***

Contents

1. Introduction

1.1 Dionex IonPac AmG-3µm C18 Columns

The Thermo Scientific Dionex™ IonPac® AmG-3µm C18 columns are specifically designed for ion-pairing reversed phase analysis of various aminoglycoside antibiotics. The stationary phase is prepared through the bonding of C18 ligand covalently onto a polymer encapsulated silica media, which ensures ultra-stability when exposed to varied conditions ranging from low pH, high temperature, organic solvents, and aqueous mobile phases. Dionex IonPac AmG-3µm C18 columns provide excellent selectivity and high resolution for the separation of various aminoglycoside antibiotics. The columns are offered in 4x150 mm PEEK (analytical column) and 4x30 mm PEEK format (guard column) formats.

Part Number	Product Description
302693	Dionex IonPac AmG-3µm C18 Analytical Column, 4 × 150 mm
302694	Dionex IonPac AmG-3µm C18 Guard Column, 4 × 30 mm

The characteristics of Dionex IonPac AmG-3µm C18 columns are summarized below:

Bonding Chemistry:	Proprietary C18
Silica Substrate:	Spherical, high-purity
Particle size:	3 µm
Surface area:	300 m^2/g
Pore size:	120 Å

2. Operation

2.1 Operating Flow Rate

Maximum operating flow rate for the Dionex IonPac AmG-3µm C18 is 1.10 mL/min, provided the column pressure does not exceed 5000 psi.

2.2 Operating Pressure

The back pressure of the column is strongly correlated to the column temperature, flow rate, and mobile phase. Submitting the column to pressures higher than 5000 psi will damage the column.

2.3 Eluent pH

The Dionex IonPac AmG-3µm C18 column has superior low pH stability because of its proprietary bonding technique. This also allows greater flexibility in the mobile phase pH ranging from pH 1 to pH 9 and therefore the ability to analyze a wide range of aminoglycosides.

2.4 Operating Temperature

The Dionex IonPac AmG-3µm C18 column is stable up to 60 ºC, even at low pH. The recommended operating temperature for aminoglycoside separation is between 30 ºC to 60 ºC.

The column may be permanently damaged if operated outside of the following operation parameter range:

pH Range:	pH 1 to pH 9
Temperature:	0 to 60°C
Pressure:	0 to 5000 psi
Flow Rate:	0 to 1.0 mL/ minute

3. Column Care

3.1 Column Storage

For short-term storage, the column can be stored in the mobile phase. For long-term storage (more than 5 days), it is recommended to store the column in 70% acetonitrile + 30% 100 mM sodium acetate, pH 5.2.

3.2 Column Washing Procedure

If tailing peaks are observed, wash the column using the following eluent conditions:

Time (min)	%A	%B	Flow Rate (mL/min)
0	80	20	0.5
4	80	20	1.0
12	80	20	1.0
12.1	50	50	1.0
20.0	50	50	1.0
20.1	50	50	0.0

Where A = 100% Acetonitrile and B = 0.1 M Sodium Acetate pH 5.2

If heptafluorobutyric acid (HFBA) or pentafluoropropionic acid (PFPA) have been used in the mobile phase for a long time, some aminoglycosides may later show tailing peaks. In this case, it is recommended washing the column using 100% Acetonitrile at 60ºC for at least 2 hours.

4. Example Applications

4.1 Analysis of Aminoglycosides using High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ED)

The Thermo Scientific ICS-5000+ System can be used for analysis of a wide range of aminoglycosides when the system is fitted with Dionex IonPac AmG-3µm C18 columns and an electrochemical detector operated in the pulsed amperometric detection mode. The Dionex IonPac AmG-3µm C18 column is developed for robust ion-pair separation of aminoglycosides. The Dionex IonPac AmG-3µm C18 column provides exceptional tolerance and stability under varied conditions ranging from low pH, high temperature, organic solvents, and aqueous mobile phases. This method can be used for the determination of aminoglycosides in applications ranging pharmacokinetics and drug stability studies, drug dissolution studies, development of pharmaceutical formulations, and residual control testing in different matrices.

Pulsed amperometric detection (PAD) is a sensitive and selective electrochemical detection method. Table 1 shows the quadruple-potential waveform detection waveform that can be used for detection of aminoglycosides.

Table 1 Quadruple Pulsed Amperometric Detection Waveform

Time (s)	Potential (V)	Integration
0.00	+0.10	
0.20	+0.10	Start
0.40	+0.10	End
0.41	-2.00	
0.42	-2.00	
0.43	+0.60	
0.44	-0.10	
0.50	-0.10	

4.2 Analysis of Etimicin and Related Impurities

The separation of Etimicin and related impurities using the Dionex IonPac AmG-3µm C18 column is shown in Figure 1.

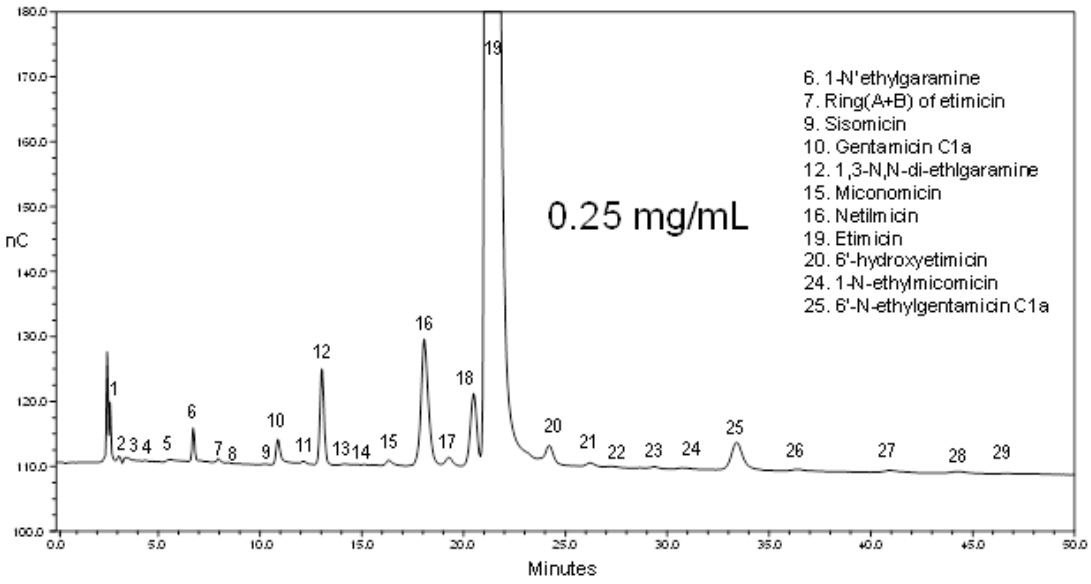
Eluent:	0.2 M TFA + 0.05% PFPA + 1.5 g/L Na_2SO4/ 4% CH_3CN (pH 3.5)
Columns:	Dionex IonPac AmG-3µm C18 analytical column (4x150 mm) and guard column (4x30 mm)
Flow rate:	0.80 mL/min
Column temperature:	35°C
Injection volume:	20 µL
Reference electrode:	Ag/AgCl
Working electrode:	Au (3 mm)
Post-column addition reagent:	0.76 M NaOH
Post-column addition flow rate:	0.30 mL/min.
Detection method:	Pulsed amperometric detection with quadruple waveform (See Table 1)
Detection temperature:	35°C
Sample:	0.25 mg/mL Etimicin

Peaks:

6. 1-N'ethylgaramine
7. Ring (A+B) of etimicin
9. Sisomicin
10. Gentamicin C1a
12. 1,3-N,N-di-ethlgaramine
15. Miconomicin
16. Netilmicin
19. Etimicin
20. 6'-hydroxyetimicin
24. 1-N-ethylmicomicin
25. 6'-N-ethylgentamicin C1a

All other peaks in the chromatogram are unknown.

Figure 1 The separation of Etimicin and related impurities using the Dionex IonPac AmG-3µm C18 column



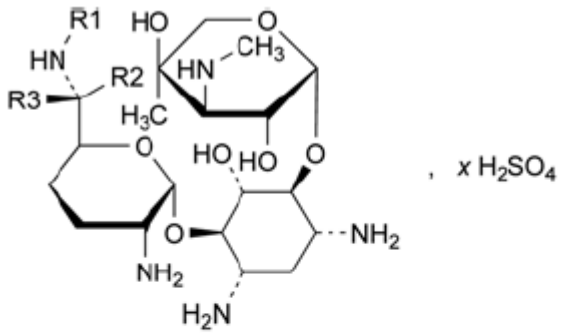
For a more general discussion of analysis of etimicin and related impurities, please review the following two references:

1. Etimicin sulfate, ChP . 2015, 1343-1344;
2. Y. Wu, W. Zhao, X. Zhu, F. Wang, M. Zhang, X. Fan, Y. Yuan, C.Hu, X. Deng and E. Adams, J. Sep. Sci., 39 (2016) 1471 – 11479.

4.3 Analysis of Gentamicin and Related Impurities

Gentamicin is a mixture of antimicrobial substances produced by Micromonospora purprea, the main components being gentamicins C1, C1a, C2, C2a and C2b (Figure 2, sulfate form).

Figure 2 Structure of Gentamicin Sulfate



Gentamicin	Mol. Formula	R1	R2	R3
C1	$C_{21}H_{43}N_5O_7$	CH_3	CH_3	H
C1a	$C_{19}H_{39}N_5O_7$	H	H	H
C2	$C_{20}H_{41}N_5O_7$	H	CH_3	H
C2a	$C_{20}H_{41}N_5O_7$	H	H	CH_3
C2b	$C_{20}H_{41}N_5O_7$	CH_3	H	H

The separation of gentamicin and related impurities using the Dionex IonPac AmG-3µm C18 column is shown in Figure 3.

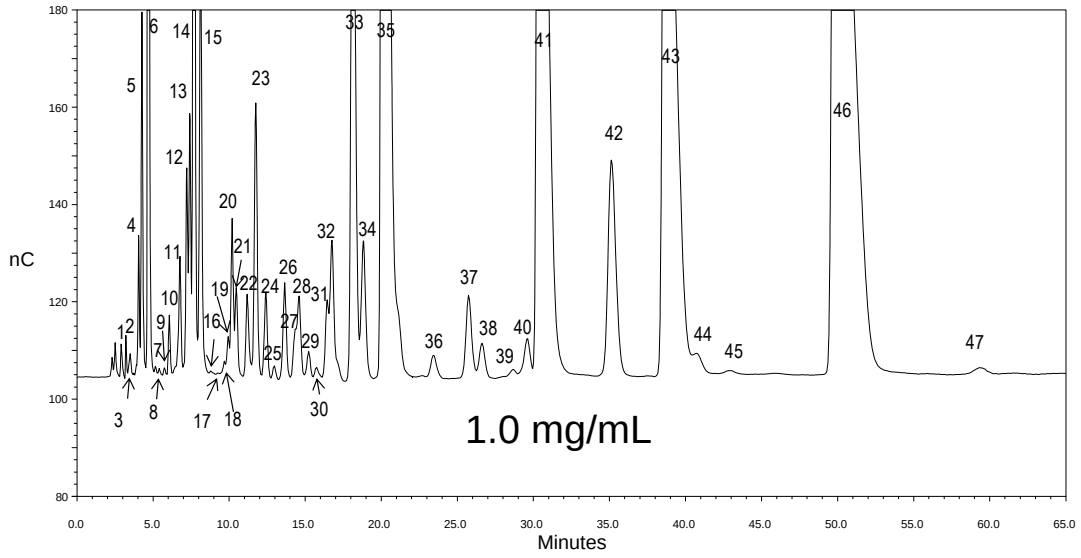
Eluent:	0.1 M TFA + 0.025% PFPA/ CH3CN (97:3, pH 2.6)
Column:	Dionex IonPac AmG-3µm C18 (Analytical: 4x150 mm)
Flow rate:	0.80 mL/min
Column temperature:	35°C
Injection volume:	20 µL
Reference electrode:	Ag/AgCl
Working electrode:	Au (3 mm)
Post-column addition reagent:	0.76 M NaOH
Post-column addition flow rate:	0.30 mL/min
Detection method:	Pulsed Amperometric Detection with quadruple waveform (see Table 1)
Detection temperature:	35°C
Sample:	1.0 mg/mL

Peaks:

33. Sisomicin;
35. Gentamicin C1a;
41. Gentamicin C2;
42. Miconomicin;
43. Gentamicin C2a;
46. Gentamicin C1;

All other peaks in the chromatogram are unknown.

Figure 3 The separation of gentamicin and related impurities using the Dionex IonPac AmG-3µm C18 column



For a more general discussion of analysis of gentamicin and related impurities, please review the following reference:

1. Gentamicin sulfate, EP 8.0, 2326-2328.

4.4 Analysis of Spectinomycin and Related Impurities

Spectinomycin is a water-soluble aminoglycoside antibiotic. It is industrially produced by fermentation of the bacterium Streptomyces spectabilis. Spectinomycin is also produced in nature by many organisms including cyanobacteria and various plant species. It is used for intravenous administration to treat infections. Spectinomycin must be analyzed and all impurities meet specified limits before a manufactured lot is used clinically.

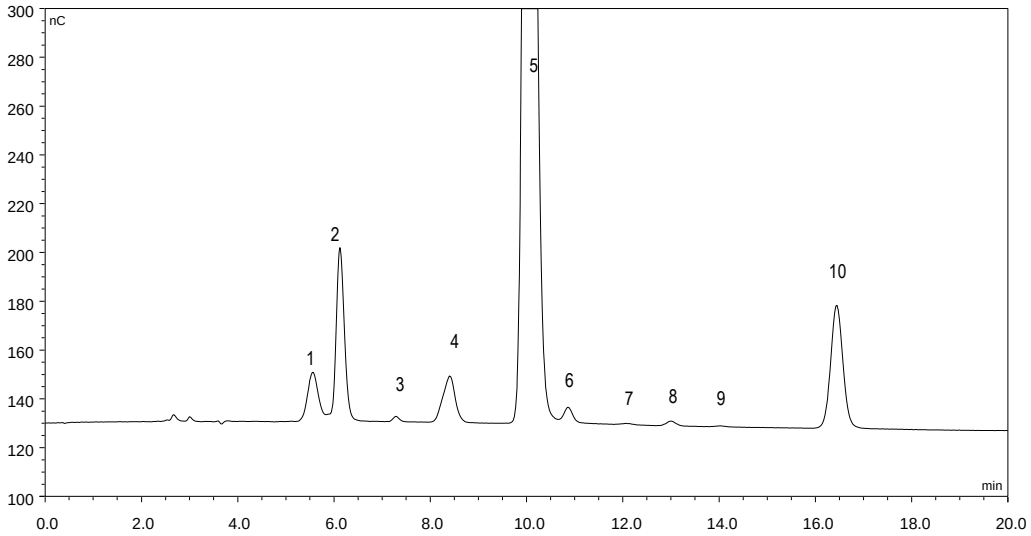
The separation of spectinomycin and related impurities using the Dionex IonPac AmG-3µm C18 column is shown in Figure 4.

Eluent:	0.1 M TFA
Column:	Dionex IonPac AmG-3µm C18 (Analytical: 4x150 mm)
Flow rate:	0.80 mL/min
Column temperature:	30°C
Injection volume:	20 µL
Reference electrode:	Ag/AgCl
Working electrode:	Au (3 mm)
Post-column addition Reagent:	0.76 M NaOH (see Appendices Section 5.1.5.2.)
Post-column addition flow rate:	0.30 mL/min
Detection method:	Quadruple Pulsed Amperometric Detection (see Table 1, Section 4.1)
Detection temperature:	30°C
Sample:	0.15 mg/mL

Peaks:
5. Spectinomycin;
10. (4R)-dihydrospectinomycin;

All other peaks in the chromatogram are unknown.

Figure 4 The separation of spectinomycin and related impurities using the Dionex IonPac AmG-3µm C18 column



For a more general discussion of analysis of spectinomycin and related impurities, please review the following reference:

1. Spectinomycin dihydrochloridum pentahydricum, EP 8.0, 3290-3292.

4.5 Analysis of Netilmicin and Related Impurities

Netilmicin is used primarily in the form of sulfate. It is a semisynthetic, water soluble aminoglycoside antibiotic obtained by chemical modification of sisomicin. It is active against both gram-positive and gram-negative bacteria.

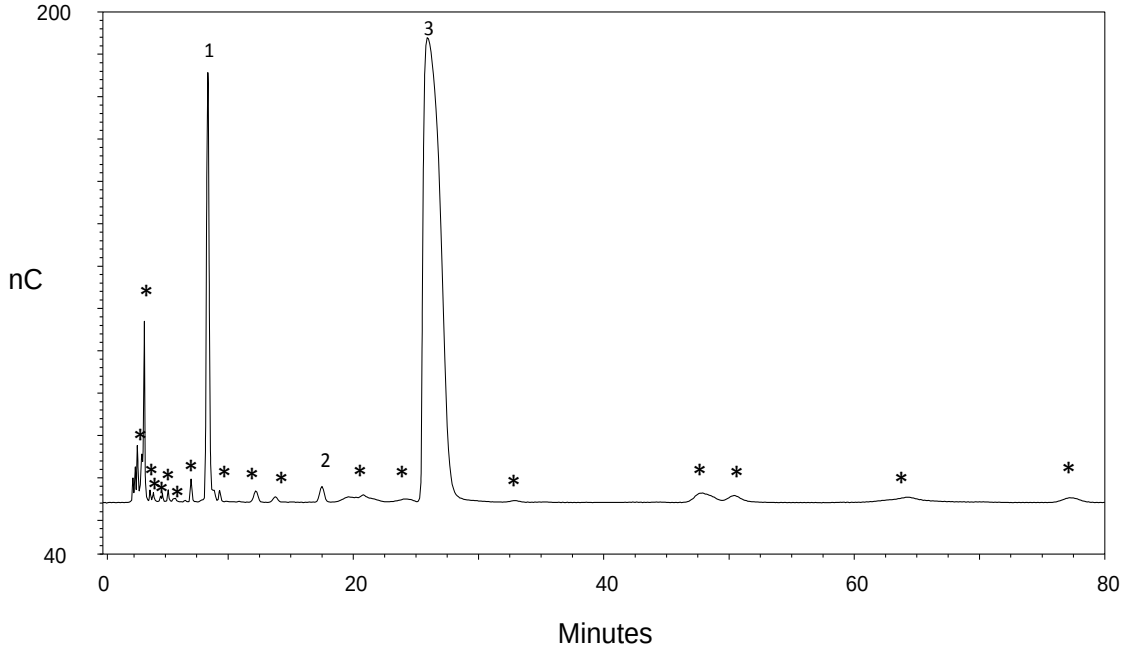
The separation of Netilmicin and its impurities using the Dionex IonPac AmG-3µm C18 column is shown in Figure 5.

Eluent:	0.2 M TFA/CH_3CN (97:3) (pH 3.0)
Column:	Dionex IonPac AmG-3µm C18 (Analytical: 4x150 mm)
Flow rate:	0.80 mL/min
Column temperature:	40°C
Injection volume:	20 µL
Reference electrode:	Ag/AgCl
Working electrode:	Au (3 mm)
Post-column addition reagent:	0.76 M NaOH (see Appendices Section 5.1.5.2.)
Post-column addition flow rate:	0.30 mL/min
Detection method:	Quadruple Pulsed Amperometric Detection (see Table 1, Section 4.1)
Detection temperature:	35°C
Sample:	0.25 mg/mL

Peaks:
1. N1-ethylgaramine;
3. Netilmicin;

All other peaks in the chromatogram are unknown.

Figure 5 The separation of Netilmicin and its impurities using the Dionex IonPac AmG-3µm C18 column



For a more general discussion of analysis of netilmicin, and related impurities, please review the following two references:

Y Cai, J. Cheng, S. Mou and P. Jandik, "Improving Sensitivity and Robustness of Chromatographic Assays", Pharmacopeial Forum 30 (4), 1501-1507;

Netilmicin sulfate, EP 8.0, 2837-2839.

4.6 References

1. "Thermo Scientific Electrochemical Detection User's Compendium", Item #: 065340-02, 2013.
2. Y. Wu, W. Zhao, X. Zhu, F. Wang, M. Zhang, X. Fan, Y. Yuan, C.Hu, X. Deng and E. Adams, "Improved liquid chromatography combined with pulsed electrochemical detection for the analysis of etimicin sulfate", J. Sep. Sci., 39 (2016) 1471 – 11479.
3. Etimicin sulfate, ChP . 2015, 1343-1344.
4. Gentamicin sulfate, EP 8.0, 2326-2328.
5. Spectinomycin dihydrochloridum pentahydricum, EP 8.0, 3290-3292.
6. Y Cai, J. Cheng, S. Mou and P. Jandik, "Improving Sensitivity and Robustness of Chromatographic Assays", Pharmacopeial Forum 30 (4), 1501-1507.
7. Netilmicin sulfate, EP 8.0, 2837-2839
8. N. H. Zawilla, J. Diana, J. Hoogmarten and E. Adams, "Analysis of Neomycin Liqiud Chromatographic Method Combined with Pulsed Electrochemical Detection", J. Chromatogra. B, 833 (2006) 191–198.
9. V. Manyanga, E. Elkady, J. Hoogmartens, and E. Adamsa, "Improved reversed phase liquid chromatographic method with pulsed electrochemical detection for tobramycin in bulk and pharmaceutical formulation", J. Pharm. Anal. 3(3), 2013, 161–167.
10. Y. Yuan, S. Chopra, X. Deng, M. Zhang, X. Fan, C. Hu, S. Jin, A. Van Schepdael, and E. Adams, "Analysis of micronomicin by liquid chromatography with pulsed electrochemical detection" J. Chromatogra. A, 1295, 2013, 90–98.
11. Dionex Corporation. "Determination of Neomycin B and Impurities Using HPAE-PAD." *Technical Note 66*, 2006, pp. 1-15.
12. Dionex Corporation. "Determination of Streptomycin and Impurities Using HPAE-PAD." *Technical Note 181*, 2012, pp. 1-12.
13. C.Ghinami, V. Giuliani, A. Menarini, F. Abballe, S. Travaini and T. Ladisa, "Electrochemical detection of tobramycin or gentamicin according to the European Pharmacopoeia analytical method", J. Chromatogra. A, 1139 (2007) 53–56.
14. R. D. Rocklin, A. P. Clarke, and M. Weitzhandler, "Improved Long-Term Reproducibility for Pulsed Amperometric Detection of Carbohydrates via a New Quadruple-Potential Waveform", Anal. Chem., 1998, 70 (8), 1496–1501.

5. Appendices

5.1 Analysis of Etimicin Using a Thermo Scientific ICS-5000+ System and Dionex IonPac AmG-3µm C18 Columns

5.1.1 Thermo Scientific Dionex ICS-5000+ System Configuration

For the analysis of aminoglycosides, the recommended Thermo Scientific Dionex ICS-5000+ system includes the following components: DP pump with degas, DC with an electrochemical detector, amperometric thin-layer cell with an 3-mm Au electrode, AS-AP autosampler fitted with 1.5 mL vial trays and 1.5 mL vial kit (glass vials with pre-cut septa), eluent organizer, four two-liter eluent bottles, and gas the regulator accessory.

5.1.2 Replacement Parts for Electrochemical Cell with Gold Electrode

Item Number	Product Description
063537	Gasket for 3 mm Au Electrode, 3 mil, PTFE
061879	Combination pH-Ag/AgCl Reference Electrode
063536	3 mm Au working electrode

5.1.3 Reagents and Purity Requirements

Obtaining reliable, reproducible and accurate results requires eluents that are free of impurities and are prepared only from the chemicals recommended below. Proper column performance may not be achieved when alternate suppliers of chemicals or lower purity water are utilized.

5.1.3.1 Deionized Water

The deionized water used to prepare eluents should be Type I reagent Grade Water with a specific resistance of 18.2 megohm-cm. The deionized water should be free of ionized impurities, organics, microorganisms and particulate matter larger than 0.2 µm. The availability of UV treatment as a part of the water purification unit is recommended.

5.1.3.2 Trifluoroacetic acid (TFA, Thermo Scientific Item # 28901)

5.1.3.3 Pentafluoropropionic acid (PFPA, 97%, Alfa Aesar Item # A12791)

5.1.3.4 Sodium Sulfate (≥99.0%, anhydrous, Sigma-Aldrich Item # 239313)

5.1.3.5 Acetonitrile (Fisher Scientific Item # A955-4)

5.1.3.6 Ethylenediamine tetraacetic acid disodium salt, dihydrate (Sigma-Aldrich Item # E4884)

5.1.3.7 Sodium Hydroxide (50% w/w, Certified Grade, Fisher Scientific Item # UN 1824)

5.1.3.8 Anhydrous Sodium Acetate (Sigma-Aldrich, Cat. No. 71183, ≥ 99.0%, or Fisher Scientific Item # BP333-500)

5.1.4 Getting Started

5.1.4.1 Important Recommendations

ALWAYS	use gloves (non-powder) when handling eluent bottles, samples or electrode cell parts. Don't touch these with your bare hands.
ALWAYS	use glass bottles for acidic eluents.
ALWAYS	use plastic bottles for alkaline eluents.
ALWAYS	use 50% NaOH solution only rather than NaOH pellets to make eluent.
ALWAYS	use dedicated glassware and disposable glass or plastic ware for volume adjustments.
ALWAYS	keep your NaOH eluent blanketed with inert gas. Prepare new NaOH eluent if left un-blanketed for more than 30 minutes.
ALWAYS	pull at least 40 mL of new eluent through the lines when changing eluent or adding fresh eluent. This will ensure that your fresh eluent is primed through the lines up to the pump heads.
ALWAYS	use pre-slit septa with the injection vials.
ALWAYS	use 20 µL or less loop size; larger loops will cause loss of resolution
ALWAYS	install and use the piston wash option
NEVER	go to the next step of the procedure if the previous has failed.
NEVER	start an installation with any of the check list items below missing (see Section 5.1.4.2. below).
NEVER	use bottled HPLC water. Do not store 18.2 megohm-cm water, always use freshly drawn water for any preparation of eluents.
NEVER	use 'communal' filtration units or filters made of unknown or unsuitable (cellulose derivatives, polysulfone) materials.
NEVER	use inlet filters; cover the ends of the eluent lines with parafilm when changing bottles.
NEVER	use METHANOL or other organic solvent as rinse fluid in the autosampler.
NEVER	use the column and/or guard column above 60 °C, 5000 psi or 1.10 mL/minute eluent flow rate

5.1.4.2 Initial Check List

These items MUST be available in your lab. The absence of any of these may compromise your analysis.

- ☐ Laboratory water unit delivering 18.2 megohm-cm water at the installation site.
  Vacuum pump available for use with the vacuum filtration units.
- ☐ Sterile-packed Nylon Nalgene Filtration Units, Funnel Size 1.0 L (VWR Cat. No. 28198-514, Fisher Cat. No. 09-740-46 or Nalgene Cat. No. 164-0020).
- ☐ Inert gas cylinder (helium or nitrogen) with a regulator valve (ca 0–200 psi at the low pressure side) and the appropriate size adaptors plus tubing.
- ☐ Etimicin standard.
- ☐ One spare Certified 3 mm Gold Electrode Item # 063536.
- ☐ One spare pH-Ag/AgCl reference electrode Item # 061879.
- ☐ One PTFE knitted reaction coil (375 µL, Item # 043700).
- ☐ One PTFE Gasket for 3 mm Au Electrode, 3 mil, Item # 063537.
- ☐ One 3-way connector.
- ☐ Powder-free, disposable gloves (at least 1 box).
- ☐ Disposable, plastic (PE) large-size (at least 20 mL) syringe for priming the pump.

5.1.5 Preparation of Eluents and Standards

The following precautions should be followed rigorously when preparing eluents and standards:

A. Always use 18 Meg-ohm water that has been filtered through a 0.2 µm Nylon filter. Avoid bacterial contamination of eluent bottles and tubing.
B. Minimize any extraneous contamination of eluents and standards. Dedicate glassware, pipettes, filtration apparatus for exclusive use in preparation of the eluents and standards only. Wear disposable, powder-free gloves whenever preparing or refilling eluents and standards.
C. Minimize the level of carbonate introduced into the eluents, especially when preparing solutions of NaOH.
D. The bacterial contamination is minimized by wearing gloves, keeping containers closed whenever possible and by ultrafiltration (filter pore size < 0.2 µm). Use ultrafiltration as indicated in the instructions for preparing the mobile phase.

5.1.5.1 Preparation of eluent for the separation of etimicin using a Dionex IonPac AmG-3µm C18 column

Below is the recommended procedure for the preparation of the eluent used for the separation of Etimicin on a Dionex IonPac AmG-3µm C18 column.

It is important to prepare the eluent concentrate in a hood, as TFA and PFPA are volatile and toxic.

5.1.5.1.1 10x Eluent Concentrate (without Acetonitrile): 2 M TFA + 0.5% PFPA + 15.0 g/L Na_2SO_4

A. In a 1.0 L glass beaker, dissolve 30.0 grams of sodium sulfate in about 600 mL of 18-megohm water.

B. Filter it through a 0.2 µm Nylon filter into a 2.0 L pre-weighted glass eluent bottle (Note: This weight will be used later.).

C. Rinse the 1.0 L sodium sulfate container with approximately 100 mL water, filtering the rinse water into the 2.0 L dedicated glass eluent bottle.

D. Weigh 456.0 g or measure 306.0 mL of TFA (d: 1.49 g/mL) in a 500 mL glass volumetric cylinder and quickly transfer into the filtered sodium sulfate solution described above.

E. Weigh 15.6 g or measure 10.0 mL of PFPA (d: 1.56 g/mL) in a 10 mL glass volumetric cylinder and quickly transfer into the sodium sulfate solution/TFA mix solution described above.

F. Weigh 320.0 g NaOH (50% w/w) in a 500 mL plastic cup and immediately transfer into the sodium sulfate solution/TFA/PFPA mix solution described above. Rinse the plastic cup with DI water and transfer into the same bottle.

This process should be done quickly to avoid carbonation of hydroxide. Shake the solution well. The reaction is exothermic, the bottle will become hot from the neutralization reaction.

G. Fill the 2.0 L eluent bottle with DI water until a net weight of 2335 g is reached (attention: the weight is more precise because the volume may change at the elevated temperature from the neutralization reaction.). The tared bottle weight is used for calculating the net weight of this 10x Eluent Concentrate.

5.1.5.1.2 Eluent for the separation of etimicin using a Dionex IonPac AmG-3µm C18 column: 0.2 M TFA + 0.05% PFPA + 1.5 g/L Na2SO4 + CH3CN (4%, v/v), pH 3.5

A. Weigh 233.5 g of the concentrate above (see Section 5.1.5.1.1) and transfer into a 2000 mL tared glass volumetric flask. Rinse the cylinder with DI water and transfer into the 2000 mL glass volumetric flask.

B. Weigh 63.2 g of Acetonitrile (d: 0.79 g/mL) and transfer into the 2.0 L glass volumetric flask.

C. Add DI water to the 2000 mL volumetric flask or until a net weight of 2033.0 g is reached.

D. Transfer it to the inert gas pressurized eluent bottle A.

5.1.5.2 Post Column Addition Reagent: 0.76 M NaOH

A. Filter about 900 mL of 18-megohm deionized water using a 1-liter Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Filter Unit with Nylon Membrane (Fisher Catalog No. 09-740-46)

B. Measure 40.0 mL in a plastic volumetric flask; immediately transfer it into the filtered DI water contained in the 1-liter bottle of the filter unit described above.

C. Add sufficient amount of DI water to the 1-lter bottle described above to bring the liquid level the 1000 mL mark. Seal the container immediately after the sodium hydroxide transfer is complete. Mix the contents of the tightly sealed container holding the 0.76 M sodium hydroxide.

D. Transfer the solution of 0.76 M sodium hydroxide to the eluent bottle for post-column addition solution immediately.

5.1.5.3 Etimicin Standards

5.1.5.3.1 Etimicin stock solution: 10.0 mg/mL

A. In a tared glass scintillation vial, dissolve 0.10 grams Etimicin in about 5 ml of 18 Meg-ohm water.

B. When it has dissolved, add enough water to bring the total weight to 10 grams. Mix well. Store up to 6 months in the freezer. In the refrigerator it is stable for weeks.

5.1.5.3.2 Etimicin standard solution: 0.25 mg/mL etimicin

A. Pipette 2.5 mL of the 10.0 mg/mL Etimicin Stock Solution into a 100 mL glass volumetric flask.

B. Weigh 11.675 g or measure 10.0 mL of the eluent concentrate in a 20 mL glass scintillation vial and transfer into the 100 mL glass volumetric flask.

C. Add DI water to fill it up to the 100 mL mark and mix well.

5.1.6 System Installation and Start-Up

5.1.6.1 System Configuration

Configure the Dionex ICS-5000$^+$ system with the AS-AP autosampler placed on the left or on the top of the DC, and the DP pump placed on the right side of the DC. Nitrogen or helium should be delivered to the eluent organizer with about 5-6 psi at each bottle. Make sure that the injection valve, column to 3-way connector, and to ED cell inlet is plumbed with red (0.005 i.d.) PEEK tubing, and that extra care is taken to minimize dead volume. Make all fluidic and electrical connections, but do not install the column yet. Instead install the yellow tubing from the Installation Kit between the injector and detector cell inlet. Assemble the electrochemical cell with the 3 mm Au Certified working electrode. Verify that the modules are communicating. In Chromeleon, create an Instrument method by choosing the "Gold, Carbo, Quad" as the detection waveform which is shown in Table 1 of the Manual.

5.1.6.1.1 System Rinse

5.1.6.1.1.1 Prior to use, RINSE a new system using the procedures described below.

It is recommended that the Dionex ICS-5000$^+$ system fluid path be rinsed using acetonitrile, 50 mM EDTA, 2 M NaOH, and DI water, separately, according to the procedures described below.

5.1.6.1.1.2 Cleaning procedure with Acetonitrile

A. Remove from the system the column and ED cell.

B. Restore liquid connection between the injector valve and waste line (the column and the ED cell have been removed).

C. Empty the contents of eluent container E1 (and E2, E3 and E4 if applicable), rinse it with 1 L of DI water and discard the water.

D. Add about 500 mL Acetonitrile to the pre-rinsed eluent bottle E1 and place all 4 eluent lines (A, B, C and D) into it.

E. Open priming valve, prime each line for 5 minutes and close the priming valve.

F. Set the pump to proportion from each of the 4 eluent lines 25% each and set the flow rate to 3 mL/min.

G. Pump for 30 minutes. During the rinsing, activate the injection valve 3 times (inject/load).

5.1.6.1.1.3 Cleaning procedure with 50 mM disodium ethylenediaminetetraacetic acid

A. Dissolve 9.3 g Ethylenediamine tetraacetic acid disodium salt dihydrate in 500 mL of deionized water. If desired, use bath sonication to speed up the process.

B. Repeat steps C thru E and G of Section 5.1.6.1.1.2 above, replacing the acetonitrile with the 50 mM disodium ethylenediamine tetraacetic acid solution.

5.1.6.1.1.4 Cleaning procedure with 2 M NaOH solution

A. Dilute 52 mL of 50% w/w NaOH (Item # 033465) to 500 mL with DI H_2O

B. Repeat steps C thru E and G of Section 5.1.6.1.1.2 above, replacing the EDTA solution with 2M NaOH solution

5.1.6.1.1.5 Rinsing procedure with DI H_2O

Repeat steps C thru E and G of Section 5.1.6.1.1.2 above, by replacing the NaOH with DI water.

5.1.6.2 Verification of System Cleanliness

Prepare a FRESH set of eluent and reagent as described in Sections 5.1.5.1 and 5.1.5.2. Open the Prime valve on the pump and set the eluent composition to 100% for each eluent. Prime each eluent line with the fresh eluent.

5.1.6.2.1 System background check

Verify the system background using the experimental conditions for etimicin as described below. Remove the separation column from the system when performing the system background check.

Eluent:	96% [0.2 M TFA + 0.05% PFPA + 1.5 g/L Na2SO4 (pH 3.5)] / 4%
Flow Rate:	0.80 mL/min
Post-column Addition:	0.76 M NaOH
Flow Rate (Post-Col. Add.):	0.30 mL/min
Column Temp.:	35°C
Detection Temp.:	35°C (not mandatory)
Working Electrode:	Au (3 mm)
Waveform:	Quadruple-potential waveform (See Table 1, Section 4.1)
Gasket:	3 mil
Reference electrode:	AgCl
Injection volume:	20 µL

Remove the separation column from the system when performing the system background check and follow the following steps:

A. Use one pump in the DP module to pump 100% of primary eluent at 0.80 mL/min and use another pump in the DP module to pump 100% of the post-column addition solution (0.76 M NaOH) at 0.30 mL/min.

B. Install a pre-determined length of PEEK yellow tubing between the injector and the detector cell to generate 1000–2300 psi backpressure

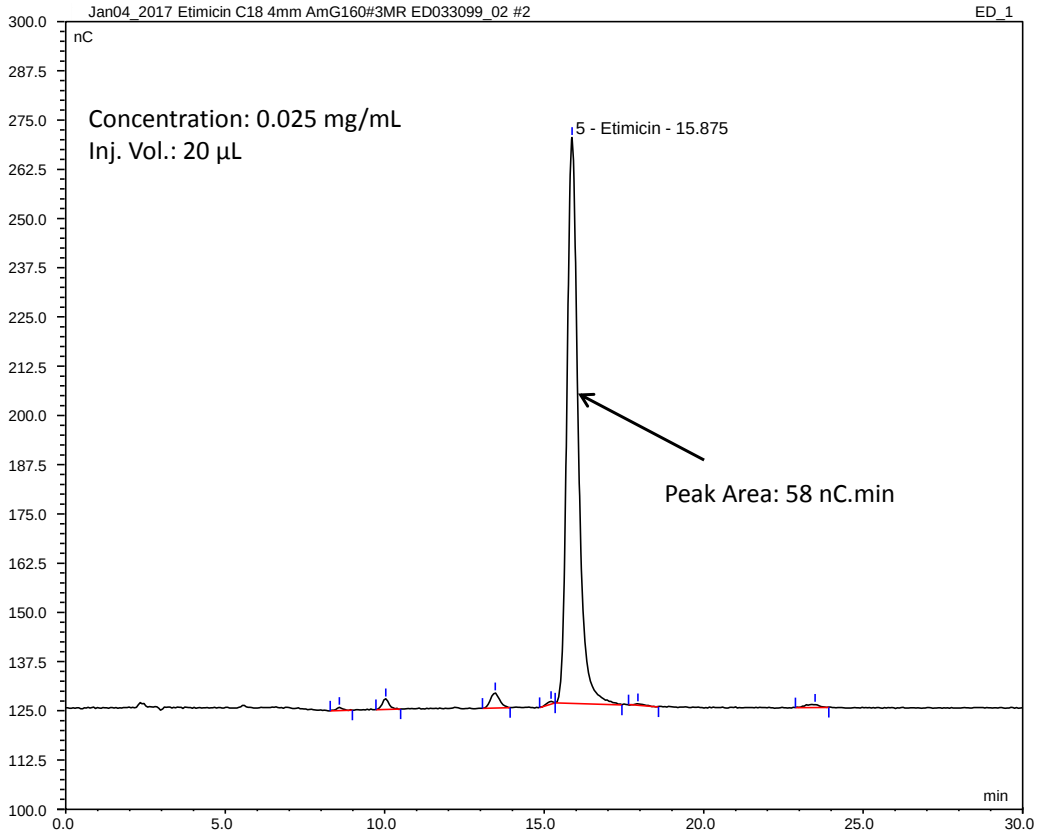
C. Confirm that the pH reading in Chromeleon is between 12.0 and 13.0.
D. With the pH within this range, turn on the cell and begin monitoring the background signal from the control panel for at least 30 min.
E. Confirm that the system ED background is 70-140 nC.

5.1.6.3 Verification of Detector Response

5.1.6.3.1 Detector response with 0.025 mg/mL Etimicin

Use the experimental conditions for Etimicin listed in Section 5.1.6.1.2 to confirm that the peak area for Etimicin is in the range of 50-70 nC.min (See Figure 6). Confirm that the %RSD for Etimicin peak area is < 3%.

Figure 6 The separation of Etimicin and related impurities using the Dionex IonPac AmG-3µm C18 column



5.1.6.4 System shutdown

As with all analyses of aminoglycosides, best reproducibility is obtained with continuous use of the system. If it is not possible to keep the system running continuously, then the system should be taken care of as described below, depending upon whether the shutdown is short-term or long-term.

5.1.6.4.1 Short-term system shutdown

Short-term system shutdown is defined as overnight or over a weekend. If the system is to be shut down for longer than 2–3 days, then follow the procedure for the long-term system shutdown.

To shut the system down short-term, eluent should be pumped continuously through the system at a very slow flow rate until the system is next ready to be used. Thermo Scientific recommends pumping both eluents (primary eluent and post-column addition eluent) through the system at 0.05 mL/min. This can be accomplished automatically by adding an extra line to your final schedule of the day, with a new method reflecting these conditions. If the system is being run manually, then these conditions should be programmed into the computer or via the front panel of the pump, when the last injection has been completed.

5.1.6.4.2 Long-term system shutdown

Long-term system shutdown is defined as longer than a weekend (5 days or longer). Follow the following procedure for long-term system shutdown.

A. Program the pump to deliver [Acetonitrile/50 mM sodium acetate (50:50, v/v, pH 5.2)].
B. Pump this solution through the columns for 60 minutes at 0.40 mL/min, and turn off the pump.
C. Remove the columns, 3-way connector and reaction coil.
D. Plug the column ends with the plugs that were in place when you received the columns and the accessories.
E. Using a union or a piece of 0.005" i.d. red PEEK tubing to replace the columns, reconnect the detector to the injection valve and rinse the entire LC system with water for 60 minutes to eliminate all traces of sulfate and carbonate which could crystallize in the check valves, lines etc.
F. Turn off the pump, remove the reference electrode and immerse it in 3 M KCl. The original "soaker" bottle in which the electrode was shipped is ideal for the storage container.
G. Disassemble the rest of the ED cell, rinse the working electrode in 18.2 megohm-cm water (wear gloves to avoid contaminating the electrode), allow it to dry and then place the electrode in a clean bag or other suitable clean, enclosed container. The titanium body can be stored in a drawer placed on a fresh towel or other type of clean surface.

5.2 Quality assurance report (QAR) and test

5.2.1 Preparation of mobile phase for quality assurance report (QAR) test

A. Prepare the QAR mobile phase to test the column under QAR conditions:
   1. 0.1 M Sodium Acetate, pH 5.2
   2. In a 2 L volumetric flask weigh in
   3. 16.408 ± 0.05 g anhydrous sodium acetate (Sigma-Aldrich, Cat. No. 71183)
   4. Add deionized water to the 2-L mark and dissolve the salt.
   5. Using a calibrated pH meter, adjust the solution to pH 5.2 by adding about 3.20 ± 0.05 g glacial acetic acid

B. QAR Mobile Phase: [70% Acetonitrile + 30% (0.1M Sodium Acetate, pH 5.2)]
   1. In a 1-L glass eluent reservoir, weigh 546 g Acetonitrile.
   2. Add 300 g of 0.1 M Sodium Acetate pH 5.2 solution (see "A" above).
   3. Mix well and degas using the ultrasonic bath (no vacuum).

5.2.2 QAR Standard Solution

A. QAR Standard Stock Standard Solution:
   1. Into a clean, dry 100 mL volumetric flask add:
      a) 15 mg of uracil
      b) 15 mg of phenanthrene
      c) 75 µL of dimethylphthalate
   2. Dissolve the standards in 100 mL of QAR test mobile phase.

B. QAR Standard Solution:
   1. Dilute 10 mL of the QAR standard stock solution to 100 mL with the mobile phase to prepare the QAR standard solution containing the following standards:

Uracil:	0.015 mg/mL
Dimethylphthalate:	0.075 µL/mL
Phenanthrene:	0.015 mg/mL

5.2.3 Set up the LC system for QAR test

A standard LC system equipped with a LC pump, a column oven, autosampler and UV detector set at 254 nm are required for the QAR test. The UV detector cell path length should be 9 to 10 mm and the system should be optimized for low dead volume; usage of small internal diameter tubing between 0.005 and 0.010" i.d. is recommended. Minimize the tubing lengths. The system should be thoroughly primed with the QAR Mobile Phase before connecting the column.

5.2.4 Conditioning the Column

Once the 4x150 mm Dionex IonPac AmG-3µm C18 column is connected and using the QAR Mobile Phase, ramp up the flow rate slowly to 0.756 mL/minute. Equilibrate the column with mobile phase for 20 minutes.

5.2.5 Operating Conditions for the QAR Test

Column:	Dionex IonPac AmG-3µm C18 (4x150 mm)
Eluent:	[70% Acetonitrile + 30% (0.1M Sodium Acetate, pH 5.2)]
Flow Rate:	0.756 mL/minute
Column Temp.:	30˚C
Inj. Volume:	4 µL
Detection:	UV, 254 nm

Peaks:

1. Uracil 0.015 mg/mL
2. Dimethylphthalate 0.075 µL/mL
3. Phenanthrene 0.015 mg/mL

Make at least 3 injections of the standard or until the chromatography is reproducible in retention times and peak efficiencies.

You should compare your chromatographic results to the QAR shipped with your column.

Below is an example of a QAR.

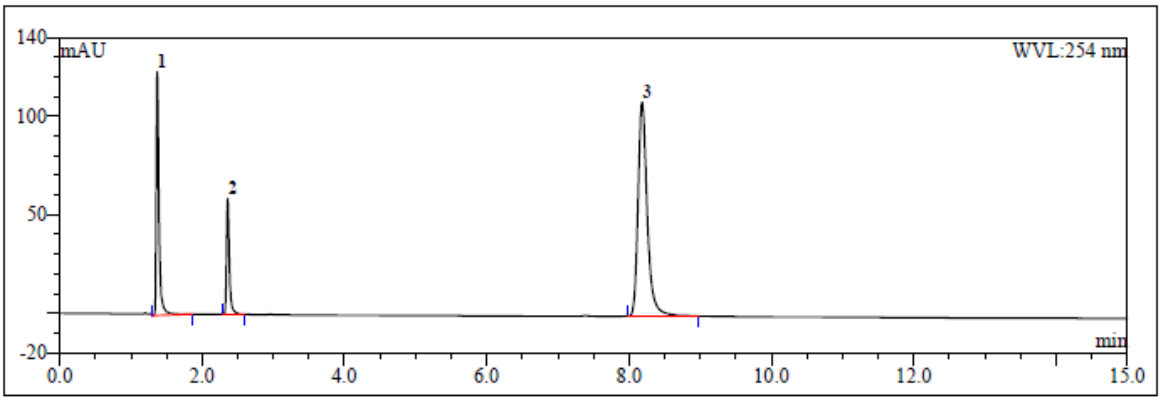
Dionex™ IonPac AmG C18
3µm (4.0 X 150 mm)
Product No. 302693

Device Monitoring Enabled
and Viper Fitting Ready

Date: 10-Apr-17 16:02
Serial No. :
Lot No. : 2015-XC-13-160

Mobile Phase: 70/30 v/v Acetonitrile/0.10 M Sodium Acetate, pH 5.20
Flow Rate: 0.756 mL/min **Temperature:** 30°C
Detection: 254 nm **Injection Volume:** 4.0 µL

Storage Solution: Mobile Phase



No.	Peak Name	Ret.Time (min)	Asymmetry (EP)	Effieciency (EP)	Concentration
1	Uracil	1.37	1.83	7597	0.015 mg/mL
2	Dimethylphthalate	2.36	1.54	19287	0.075 µL/mL
3	Phenanthrene	8.18	1.32	21619	0.015 mg/mL

QA Results:

Analyte	Parameter	Specification	Results
Phenanthrene	Retention Time	7.43-9.08	Passed
Phenanthrene	Efficiency	>16,200	Passed
Phenanthrene	Asymmetry	1.00-1.55	Passed
	Pressure	<=1980	1480

Production Reference:

Datasource: Silica
Directory: Silica3\Silica3_2
Sequence: 1517061_VAL_4x150_3µ_AM
Sample No: 8

6.80 SR15 Build 4656 (243203) (Demo-Installation)

Chromeleon™ Thermo Fisher Scientific